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A Simplified Fabrication Technique for Cellularized High-Collagen Dermal Equivalents

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Abstract

Human autologous bioengineered skin has been successfully developed and used to treat skin injuries in a growing number of cases. In current clinical studies, the biomaterial used is fabricated via plastic compression of collagen hydrogel to increase the density and stability of the tissue. To further facilitate clinical adoption of bioengineered skin, the fabrication technique needs to be improved in terms of standardization and automation. Here, we present a one-step mixing technique using highly concentrated collagen and human fibroblasts to simplify fabrication of stable dermal equivalents. As controls, we prepared cellularized dermal equivalents with three varying collagen compositions. We found that the dermal equivalents produced using the simplified mixing technique were stable and pliable, showed viable fibroblast distribution throughout the tissue, and were comparable to highly concentrated manually produced collagen gels. Because no subsequent plastic compression of collagen is required in the simplified mixing technique, the fabrication steps and production time for dermal equivalents are consistently reduced. The present study provides a basis for further investigations to optimize the technique, which has significant promise in enabling efficient clinical production of bioengineered skin in the future.

Keywords: Skin, Automation, Tissue Engineering, Bovine Collagen, Plastic Compression

1. Introduction

Autologous tissue-engineered skin grafting is a promising approach for the treatment of skin injuries, such as burns or scars [1,2]. Such skin grafts facilitate wound healing and restore skin function while overcoming donor site limitations and immune rejection [3,4], and have been successfully used in initial clinical studies [5]. However, the fabrication of dermal equivalents is complex and time consuming using current fabrication techniques, and simplification could result in higher standardization and lower costs, which is a valuable aspect for commercial translation.

Regarding the process of skin tissue engineering, biopsied skin cells are isolated and rapidly expanded in vitro. Combined with a scaffold biomaterial, the cells are formed into biomimetic tissue and transplanted back onto the patient [6].

Over the last few decades, various scaffold biomaterials have been established for creating dermal equivalents of bioengineered skin [3,7–9], and are currently being applied in clinical practice [10–15]. In the present study, we focused on the use of natural biomaterials, namely collagen, due to successful outcomes in clinical settings [5,11]. In skin substitutes, the scaffolds mimic the extracellular matrix (ECM) and provide mechanical stability to the graft, which is

essential for convenient surgical handling. However, several products lack handling properties suitable for a practical clinical application [16], and are based on complex and intricate fabrication techniques that reduce repeatability and standardization. To overcome these limitations, Brown et al. [17] have reported a method that increases the stability of collagen scaffolds. To achieve high mechanical stability, collagen hydrogel is compacted using external pressure by displacing water and increasing the final collagen density. Braziulis et al. [16] have improved this technique and used dermal scaffolds in clinical applications [5].

Furthermore, automated biofabrication is crucial in improving standardization and production robustness, as well as cost-effectiveness. In addition, a simplification of the fabrication process reduces complexity in the automation setup. One promising approach toward automated biofabrication is 3D bioprinting [18], particularly bioprinting of the skin, which has been successfully used in vitro and in animal models. A review of this approach was recently published [8]. Several different biomaterials are currently being developed for 3D bioprinting [19,20]. However, natural biomaterials, such as collagen, are typically used at an extremely low concentration [21,22]. As a result, additional treatment, such as chemical crosslinking, is required, or a mechanically fragile tissue is obtained.

In the present study, we aimed to combine the best of both worlds; using an established natural biomaterial to result in high mechanical stability and simplifying fabrication.

We developed a fabrication technique inspired by extrusion mixing, in which collagen and fibroblasts were continuously extruded through a 3D printed mixing chamber into a culture well to form in vitro dermal equivalents. This technique for creating cellularized dermal equivalents requires only one fabrication step and is capable of processing highly concentrated collagen solutions that allow compression to be avoided. As controls, three collagen compositions were prepared manually; 5 mg/mL non-pepsinized collagen I, a standard used for in vitro skin models [23], 25 mg/mL non-pepsinized collagen, and 25 mg/mL pepsinized collagen. The dermal equivalents fabricated using the simplified mixing technique were investigated to evaluate cell viability and compared to the control gels in terms of stability/pliability, cell distribution, and cell morphology. We found that the resulting dermal equivalents showed good cell viability, cell morphology, and spatial distribution, and had fine handling properties. Our results indicate that the use of this biomaterial and fabrication process is feasible for the simplification of the production of clinically applicable skin grafts.

2. Materials and Methods

2.1. Cell Culture

Human foreskin samples were harvested from patients aged between 6 and 16 years old. Approval was obtained from the Ethics Committee of the Canton Zurich, Switzerland. Parents or patients gave informed consent to use the skin samples. Skin biopsies were used for the isolation of dermal fibroblasts as described previously [16]. Skin samples were minced into small pieces (10 mm^2) and digested for 15–18 hours at 4°C in 12 U/mL dispase in Hank's balanced salt solution containing 5 mg/mL gentamycin. The epidermis and dermis were mechanically separated using forceps. The dermal tissue was digested in 2 mg/mL collagenase (Clostridiopeptidase A) for 60 minutes at 37°C. Isolated cells were seeded on cell culture dishes containing fibroblast growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum [FCS], 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 mg/mL gentamycin) and allowed to attach overnight (37°C, 5% CO_2). Fibroblasts were further cultured until Passage 5 in the medium mentioned above at 37°C and 5% CO_2. The transfection of human fibroblasts with a green fluorescent protein (GFP)-expressing cDNA sequence was performed as described previously [24].

2.2. Manual preparation of dermal equivalents

Three different collagen compositions were manually mixed with human-derived skin fibroblasts to form a dermal equivalent. Non-pepsinized bovine collagen Type I at concentrations of 5 mg/mL and 25 mg/mL, as well as pepsinized bovine collagen Type I at a concentration of 25mg/mL in 0.063M acetic acid were used. A preparation of 5 mg/mL non-pepsinized bovine collagen Type I served as control [16]. The acidic solutions of bovine collagen Type I were neutralized on ice via addition of 0.5 M NaOH. Multiple dermal equivalents of each composition were formed manually with a spatula in Corning Transwell six-well inserts with a diameter of 24 mm (Figure 1A, n = 6). The green fluorescent protein (GFP)-expressing human dermal fibroblasts were seeded at 3×10^6 cells per hydrogel, with a mixing time of approximately 30 seconds each. Fibroblast growth medium was added after the hydrogel cured and the preparation was cultured for seven days at 37°C in a humidified atmosphere containing 5% CO_2. The medium was renewed every 2–3 days. The collagen was acquired from Symatese (Chaponost, France). All other compounds were obtained from Invitrogen (Basel, Switzerland).

2.3. Simplified technique for high-collagen dermal equivalents

For the fabrication of a dermal equivalent using the molding technique, a simple extrusion module composed of two plastic syringes (3 mL and 5 mL) and a disposable 3D-printed mixing chamber was designed (Figures 1B–D). The mixing chamber was designed using the Siemens NX10 Software (Siemens PLM Inc., USA) and printed on a Form 2 SLA Printer (Formlabs Inc., USA) with clear resin

(GPCL02) and then sterilized using 70% ethanol. Figure 1 D illustrates the fluid path. The cell medium is guided into a barrel-shaped cavity with openings toward the front. To reduce stress on the cells, the viscous collagen is guided above and below the barrel shape, and the cells exiting the barrel openings are "sandwiched" in between the top and bottom collagen streams (as illustrated in the side view). Furthermore, as visible in the top view, the mixture is guided through a tunnel with distributed mixing columns. The complete molding module was intended for single use.

An acidic solution of highly concentrated (25 mg/mL) non-pepsinized bovine collagen Type I was prepared in a sterile 5 mL syringe and kept on ice until use. A total of 3 x 10^6 (GFP)-expressing human dermal fibroblasts were suspended in DMEM in a second sterile 3 mL syringe and shortly before mixing, a buffer containing 200 mM HEPES, 0.32 M $NaHCO_3$, and 0.15 M NaOH was added as described previously [16]. The loaded syringes were connected to the 3D-printed mixing chamber and sealed with O-rings. The content was injected through the mixing chamber and distributed into multiple Transwell® six-well inserts. The first 1 mL aliquot of the mixture was discarded to ensure homogeneity. A plastic ring in the insert helped distribute the gel (not shown). The extrusion time per gel was less than 15 seconds. The dermal equivalents were left at room temperature for 15 minutes to allow the hydrogels to cure before the fibroblast growth medium was added. Culturing was done for seven days at 37°C in a humidified atmosphere containing 5% CO_2.

Homogenous mixing with the nozzle was initially assessed via visual observation of colored water (blue and red coloring; Dr. Oetker, Bielefeld, Germany) Additionally, to account for high viscosity of the biomaterial, mixing was assessed visually with the described collagen and buffer, both stained with phenol red (Sigma Aldrich, St. Louis, MI, USA). For Figures 3 C and D, color saturation of the pictures was increased with Apple Preview (Apple, Cupertino, CA).

2.4. Analysis of spatial distribution of cells and cell viability

Each hydrogel was analyzed at three different depths with fluorescence microscopy. The morphology and spatial distribution of the cells were observed. This optical study occurred for seven days and in three layers. The layers are shown in Figure 2. The dermis equivalents were initially examined under the microscope and at 2, 6, 24 and 48 hours (not shown), as well as at day seven after the mixing process. Fluorescence microscopy pictures were acquired using a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope equipped with an FITC filter or a Nikon SMZ1500 stereo microscope with FITC filters (Nikon AG, Egg, Switzerland; Software: Nikon ACT-1 version 2.70). The depth positions were initially set via the focus, marked, and then repositioned. Microscopic and macroscopic images were post-processed using Photoshop 10.0 (Adobe Systems Inc., Munich, Germany) for cropping and contrast, color, and background enhancements.

For cell viability assay following the simplified mixing technique, dermal equivalents were fabricated as described above in a volume of 50 µL with 10,000 primary human dermal fibroblasts each and extruded into a commercially available 96-well plate. The gels were suspended in fibroblast growth medium, incubated for 1.5 h, and collagenase (4 mg/mL) was then added. The gels were incubated for an additional 1.5 h until the gel was digested. Cell viability was measured 3 h after fabrication using a trypan blue exclusion assay and an automated cell counter (Countess II, Invitrogen). In addition, cell viability was observed after 24 h via resazurin reduction assay (CellTiter-Blue®, Promega, Madison, Wisconsin, USA; similar to alamarBlue™). A total gel volume of 50 µL with 5,000 fibroblasts were either extruded using the simplified mixing technique or manually mixed (as positive control), as described above, into a 96-wells plate and suspended in fibroblast growth medium. Non-cellularized gels were added as negative controls. After 24 h of incubation, 10 µL of CellTiter Blue® dye was added and incubated for another 4 h. Fluorescence measurements were obtained with a plate reader (BioTec Synergy HT, Winooski, Vermont, USA) at 530/590 nm. Because CellTiter Blue® is nontoxic, the assay was conducted on the same samples again 72 h after fabrication for an indication on cell proliferation.

2.5. Statistical analysis

The microscopy images were analyzed automatically with MATLAB (MathWorks Inc., Natick, USA). The images were *binarized* (translated into black and white) with a sensitivity parameter of 0.8 using the function *IM2BW* [25]. The images were further scanned with circle detection (function: *imfindcircles* [26]) with an edge threshold parameter of 0.15. This function counted the number of cells in each image. An average of six images was taken at upper, middle, and bottom depths respectively and approximately 650 cells were counted. The numbers were then manually transferred to Excel (Microsoft, Redmond, USA).

2.6. Analysis of pliability

For convenient handling, a prerequisite of dermal equivalents is their mechanical stability. After seven days of cultivation, the dermal equivalents were taken out of the Transwell inserts and mechanically manipulated with forceps. Their robustness was evaluated with a focus on folding and ripping when taken out of the insert, and compared to previously reported results [16].

3.Results

In the present study, we used a highly concentrated collagen biomaterial and a simplified mixing technique (Figures. 1 B–D) to produce stable cellularized dermal

equivalents (Figure. 2 A) and compared them to dermal equivalents produced manually using different collagen compositions (Figures. 2 B–D).

The mixing nozzle achieved a homogenous mixture of the colored water, and the color of the phenol red-stained components showed neutralization of the mixture toward the nozzle tip (Figures. 3 A–D).

The fabrication of dermal equivalents with the simplified mixing technique proved to be very fast and allowed the production of three ø24 mm gels in succession in only 15 seconds each. The fabricated gels formed well in the insert and showed homogenous opaqueness, except for a slight increase in collagen density in the center where the nozzle was placed. The hydrogels reached the walls of the Transwell insert, thereby obtaining a uniform shape (circle) and size (ø24 mm). The stability of the hydrogels was particularly high; no folding or ripping occurred when they were handled with the forceps (Figure. 2 A). Our designed and 3D-printed mixing nozzle provided sufficiently homogenous mixing (Figures. 3 A, B).

To investigate the biological characteristics of the prepared gels, the viability of the processed fibroblasts was measured 3 h after fabrication and was found to be 91.7% ± 5.1% (n=30). Fluorescent cell viability analysis after 24 h showed a slightly higher viability (mean: 6% high, $p = 0.002$, two-tailed *t*-test, $n = 30$) for the fibroblasts with the simplified mixture than in the manually fabricated control. Similar results after 72 h (mean 7%, $p=0.003$) further indicate positive cell proliferation. Microscopic cell morphology analysis showed normal elongated fibroblasts with similar morphology as in the 5 mg/mL control gels over seven days of culture in the upper, middle, and bottom layers (Figures. 2 A‘–A‘‘ and Figures. 2 B‘–B‘‘ respectively). When comparing the distribution of the cells throughout the gel, no significant difference was found between simplified mixing and manual preparation in the number of cells in the three different layers initially after preparation (both 25 mg/mL non-pepsinized collagen, $p > 0.05$, two-tailed t-test, shown in Figure. 3C).

The control gels prepared manually with the standard composition of 5 mg/mL showed a transparent hydrogel with a consistent reddish color with fibroblasts found throughout all layers of the gel (Figures. 2B–B‘‘‘, after seven days in culture). However, these dermal equivalents were very fragile when handled manually. The hydrogels folded and ripped easily when manipulated with forceps and their removal from the six-well insert was delicate, with a high probability of being destroyed.

The gels formed manually with the increased collagen concentration of 25 mg/mL non-pepsinized collagen (as in the mixing technique) formed an opaque gel with high mechanical stability; however, the collagen gelled very quickly and clumping occurred in the mixing process, which formed bulges on the surface. The fibroblasts were found in all layers of the gel (Figures. 2 C–C‘‘‘).

The pepsinized collagen composition of 25 mg/mL showed the lowest homogeneity and stability, with both transparent as well as opaque areas (Figures. 2D), and most gels ruptured when manipulated with forceps. We further observed an inhomogeneous distribution, with most of the fibroblasts at the bottom of the gel on the insert membrane.

4.Discussion

In the present study, we hypothesized that it is feasible to accelerate the fabrication of stable dermal equivalents with the use of highly concentrated collagen and a simplified mixing technique. We developed a mixing technique that allowed for the fabrication of dermal equivalents in under 15 seconds, which is considerably faster than the current approach of plastic compression [16,17].

The dermal equivalents (Figure. 2A) were compared to dermal substitutes prepared using an established manual fabrication technique (Figures. 2B–C). The equivalents were analyzed in terms of viability, spatial distribution, and handling properties.

The simplified mixing technique created robust and pliable dermal equivalents, with viable cells homogenously distributed throughout the hydrogel. After the mixing process in vitro, the fibroblasts showed a typical phenotype throughout seven days of culture. Pliability and handling were comparable with the high concentrated dermal equivalent controls and with earlier results fabricated with plastic compression [16]. Both morphology and spatial distribution of the fibroblasts were comparable with those in traditionally prepared dermal equivalents (Figure. 2B).

Viability after our mixing process was high, with 91.7% viable cells compared with 82.3% in a previous study that used plastic compression [17]. This is a positive indication; however, a reliable viability must be compared in a specific comparative study with identical cells and viability assays. After 24 h and 72 h, the viability and proliferation showed comparable results for the manually prepared gels and the cells processed using the mixing nozzle. This is another positive indication for simplified mixing feasibility. However, several factors might influence this test, such as the diffusion of the dye into the gel and the air introduced via manual mixing.

The use of the 3D printed mixing nozzle allowed us to quickly implement our idea and provided sufficient mixing and cell viability. In addition, commercially available mixing nozzles will be considered for future use because they are readily available, can be acquired pre-sterilized, and provide a cost-effective alternative (e.g medmix L-System, Medmix, Rotkreuz, Switzerland) However, cell viability, mixing, and implementation using these nozzles needs to be investigated.

The use of 25 mg/mL non-pepsinized biomaterial in combination with the mixing technique we developed represents potential improvement in the commercial fabrication of cellularized dermal equivalents, because it reduces the process to a single step allowing for simpler and

more efficient scale-up, which is important for the production of large numbers of tissues and commercial exploitation [27]. Recently, the importance of scalability has been observed in the area of electrospinning [28]. For clinical applications, the biomaterial could be pre-prepared in the syringe with the mixing nozzle and stored until needed.

The biomaterial and mixing technique described here could also be beneficially implemented in combination with automation techniques like 3D bioprinting or injection molding. In the extrusion printing of high-collagen dense constructs, cells need to be mixed thoroughly (e.g., 40 aspiration cycles) with the scaffold material used in advance to create the bioink [29,30]. In our mixing technique, the biomaterial is only mixed gently with the cells at the moment of extrusion.

Gel contraction, known to occur in fibroblast-containing collagen gels [31], was not monitored. Slight contraction was observed toward the end of the seven-day culture period in some of the 5 mg/mL dermal equivalents, but not in the 25 mg/mL non-pepsinized gels. Further investigation of the influence of our protocol on gel contraction needs to be conducted.

In general, the experimental setup of the one-step mixing technique allowed us to test fundamental principles. Some aspects still need to be improved for high-quantity production using the mixing technique. The production should be further actuated by a motor, e.g. a syringe pump, to investigate and define crucial parameters for standardization of the automated process. In relation to this, a detailed study on the optimal mixing nozzle design, mechanical stability testing, and viability and cell proliferation must be performed in the future.

It should also be noted that we solely focused on the production of the dermal equivalent. An automated application using keratinocytes to create the epidermis and to fabricate a bioengineered dermo-epidermal skin substitute is still required.

Future improvements in the mixing technique are intended to enable fully automated production of dermo-epidermal skin substitutes, which can then be compared to current state-of-the-art products. Our mixing technique has remarkable innovative potential because of its simplicity, ease-of-use, distinctive robustness, and cost-effectiveness in automation and standardization of the fabrication of bioengineered skin substitutes.

5.Conclusion

In the present study, we introduce a biomaterial in combination with a simplified automatable one-step mixing technique to fabricate dermal equivalents with high structural stability within seconds. We show that one-step mixing of highly concentrated collagen with dermal fibroblasts is feasible, with promising results in terms of cell viability, spatial distribution, and handling properties. The mixing technique is faster than the traditional dermal equivalent production and is amenable for efficient automation.

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Figures:

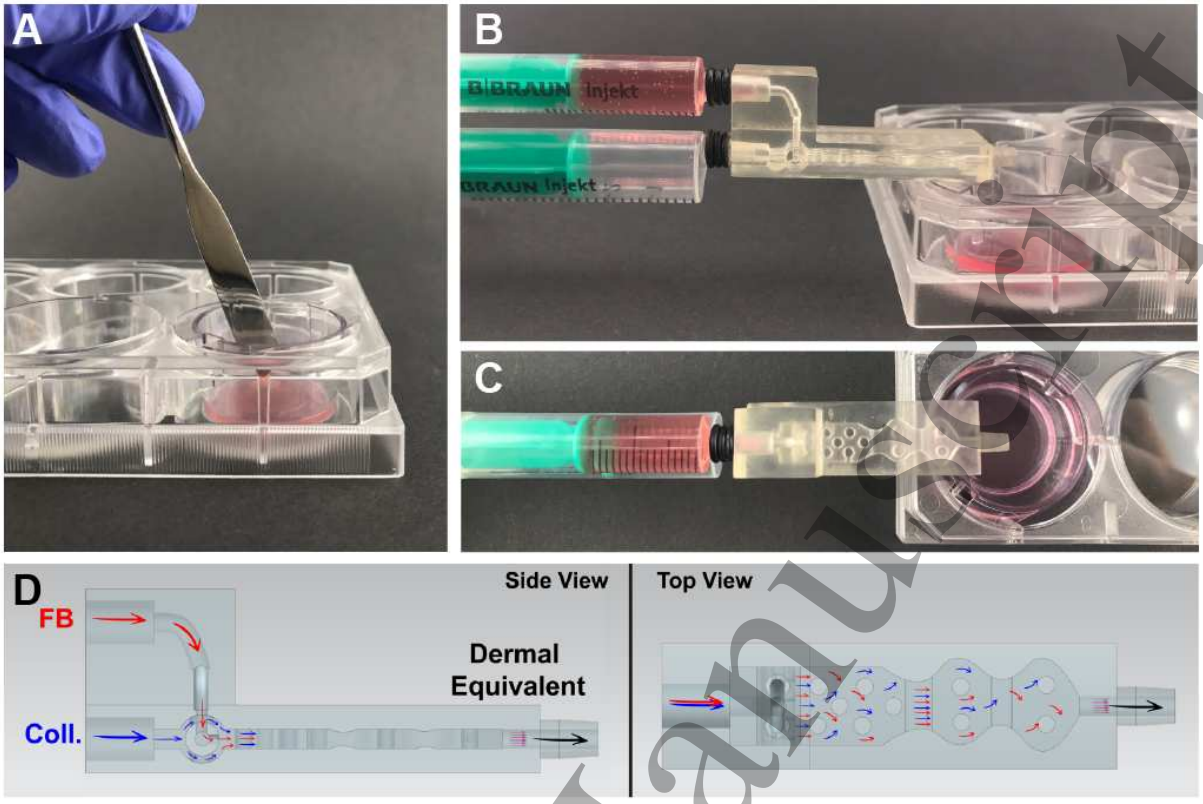

Figure 1. Two different production techniques are illustrated. **(A)** Manual production: manual spatula mixing of green fluorescent protein (GFP)-expressing fibroblasts suspended in buffer and collagen**. (B, C)** Mixing technique: a simple extrusion module composed of two plastic syringes of different sizes, and a disposable 3D-printed mixing chamber was developed. GFP-expressing fibroblasts suspended in buffer and collagen were loaded in separate syringes, both connected to a 3D-printed mixing chamber. The contents were injected via the mixing chamber, mixed, and distributed into multiple 24-mm Transwell six-well filter inserts. **(D)** The 3D-printed mixing chamber is illustrated with the Siemens NX 10/Adobe Illustrator software. Illustrative fluids were used for better image visibility.

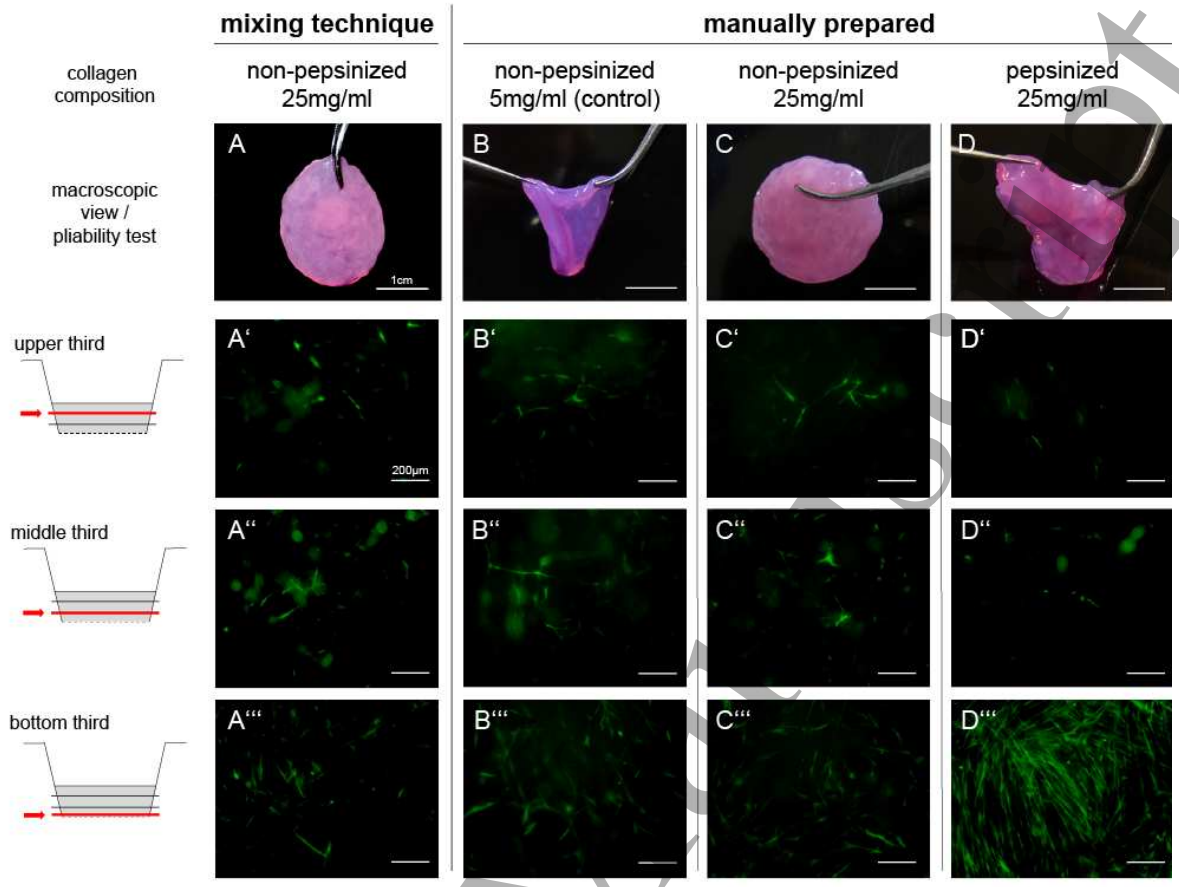

Figure 2. Investigation of the influence of high collagen concentration and the simplified mixing technique on hydrogel stability and biological characteristics. **(A–A“‘)** Dermal equivalents prepared using the mixing technique. **(A)** Macroscopic view of a highly concentrated non-pepsinized bovine collagen type I (25 mg/mL) hydrogel showing quality and fine handling properties. **(A‘–A“‘)** Fluorescence microscopy of green fluorescent protein (GFP)-expressing fibroblasts showing typical fibroblast morphology and distribution in the upper, middle, and bottom areas of the highly concentrated non-pepsinized hydrogel. **(B–B“‘)** Manually prepared dermal equivalents. **(B)** Macroscopic view of a non-pepsinized low-concentration bovine collagen type I (5 mg/mL) control hydrogel showing quality and poor handling properties. **(B‘–B“‘)** Fluorescence microscopy of GFP-expressing fibroblasts showing typical fibroblast morphology and distribution in the upper, middle, and bottom areas of the non-pepsinized low-concentration hydrogel. **(C–C“‘)** Manually prepared dermal equivalents. **(C)** Macroscopic view of a non-pepsinized high-concentration bovine collagen type I (25 mg/mL) control hydrogel showing quality and good handling properties. **(C‘–C“‘)** Fluorescence microscopy of GFP-expressing fibroblasts showing typical fibroblast morphology and distribution in the upper, middle, and bottom areas of the non-pepsinized high-concentration hydrogel. **(D–D“‘)** Manually prepared dermal equivalents. **(D)** Macroscopic view of a pepsinized high-concentration bovine collagen type I (25 mg/mL) control hydrogel showing quality and poor handling properties. **(D‘–D“‘)** Fluorescence microscopy of GFP-expressing fibroblasts showing fibroblast morphology and non-equal distribution in the upper and middle areas with high distribution in the bottom area of the pepsinized high-concentration hydrogel. All images were obtained on day 7. Scale bar: A–D, 1 cm; all other pictures, 200 µm.

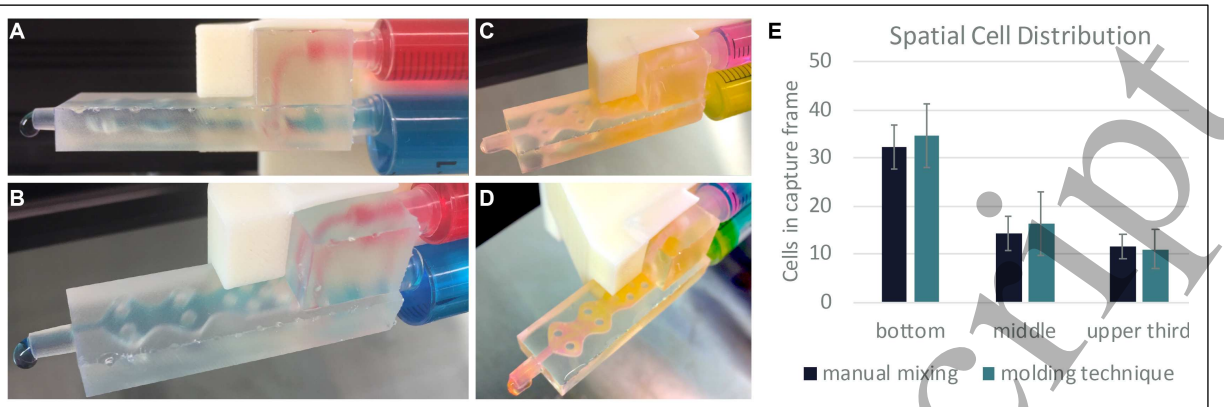

Figure 3. Colored mixing assessment and statistical analysis. **(A, B)** Side and top views of the mixing nozzle while mixing colored water. The individual syringes were filled with red or blue colored water for better visibility, and the exiting droplets showed a uniform violet color. (**C, D**) In addition, to account for the high collagen viscosity, both the described high concentrated collagen (25mg/mL) and buffer solution were colored with phenol red. The mixture shows progress from yellow to rose color toward the tip, which is an indication of the ongoing pH neutralization. **(E)** To assess the cell distribution in the vertical direction, the microscopy images of the high-concentration collagen dermal equivalents were analyzed. The cells were counted in the microscopy capture frame at three levels in the hydrogels using the developed mixing technique or manual preparation. The bars show the number of visible cells in the corresponding layers and the standard deviation illustrating the comparability of the techniques.